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Evidence for Phage-Mediated Gene Transfer among *Pseudomonas aeruginosa* Strains on the Phylloplane

SARANGA P. KIDAMBI, STEVEN RIPP, AND ROBERT V. MILLER*

Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma 74078

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As the use of genetically engineered microorganisms for agricultural tasks becomes more frequent, the ability of bacteria to exchange genetic material in the agricultural setting must be assessed. Transduction (bacterial virus-mediated horizontal gene transfer) is a potentially important mechanism of gene transfer in natural environments. This study investigated the potential of plant leaves to act as surfaces on which transduction can take place among microorganisms. *Pseudomonas aeruginosa* and its generalized transducing bacteriophage F116 were used as a model system. The application of *P. aeruginosa* lysogens of F116 to plant leaves resulted in genetic exchange among donor and recipient organisms resident on the same plant. Transduction was also observed when these bacterial strains were inoculated onto adjacent plants and contact was made possible through high-density planting.

Biotechnology is increasingly being applied in the solutions for agricultural and other environmental problems. Often these solutions involve the development of a genetically engineered microorganism able to make a product capable of improving crop production or remedying contaminated environments. Agricultural uses of engineered products include production of nutrients in the form of biological fertilizers and protection of crop plants from predators with biological insecticides. Biotechnological solutions often require the release and dispersal of a genetically engineered microorganism to the surfaces of crops and other plants. The estimation of both the efficacy and the potential risk associated with the use of genetically engineered microorganisms requires an understanding of the ecology of microorganisms associated with the phylloplane. One important question which must be addressed is whether plant leaf surfaces have the potential to support horizontal genetic transfer among phylloplane microorganisms. However, of the three most common methods of bacterial gene transfer (conjugation, transduction, and transformation), only conjugation has been investigated as it occurs on plant surfaces (6).

Although not yet studied with regard to its occurrence on the phylloplane, transduction (virus-mediated gene transfer) has been demonstrated to occur at significant levels in both aquatic environments (24, 26, 27) and soils (8), and lysogens of temperate bacteriophages have been shown to be the most likely source of transducing bacteriophages in the environment (23).

Many plant-associated bacteria are widely believed to harbor lysogenic bacteriophages (6). Many of these temperate bacteriophages have been shown to carry out transduction in the laboratory setting (15). Lysogens of many bacterial species respond to exposure to UV radiation in the 290- to 320-nm range (i.e., UV B) by the induction of lytic production of these bacteriophages (4). However, bacterial populations on leaves are not uniform (2, 10). This may lead to spatial separation of genetic donors, genetic recipients, and transducing bacteriophage particles, reducing or eliminating the possibility of gene exchange. Thus, it seemed prudent to investigate whether genetic exchange mediated through bacteriophages (i.e., transduction) can take place on plant leaf surfaces. Our objectives were to determine (i) whether the lamellar surfaces of plants could act as a support on which transduction can take place and (ii) whether migration of microbes from plant to plant (when adequate contact is made possible) could lead to transduction of genetic material.

For these studies we used *Pseudomonas aeruginosa* and its well-characterized generalized transducing phage F116 (16, 22). *P. aeruginosa* has been shown to be a plant epiphyte and an opportunistic plant pathogen (1, 3, 17, 25). Such plant-bacterium interactions are common among the pseudomonads. For instance, *Pseudomonas syringae*, which is a pathogen of many plants, can survive on others as an epiphyte without causing any detectable disease symptoms (5, 18). Spontaneous induction of phage particles from *P. aeruginosa* lysogens has been reported to occur in the environment (14, 26, 27). The genetics of *P. aeruginosa* has been well documented (11, 12), and the F116-*P. aeruginosa* transduction system has been successfully used to demonstrate transduction of both plasmid and chromosomal DNAs in aquatic environments (24, 26, 27) and has allowed us to draw analogies between the two environments, i.e., aquatic and phylloplane.

MATERIALS AND METHODS

Bacterial strains, plasmid, and bacteriophage. The bacterial strains used in this study were *P. aeruginosa* PAO1, a prototrophic strain; PAO515 (*met-9011 amiE200 nalA5*), a nalidixic acid-resistant lysogen of the generalized transducing bacteriophage F116 (16, 22, 26); and RM2140. Strain RM2140 is a derivative of PAO1 containing the plasmid Rms149 (26). Rms149 is a low-copy-number, Tra^- Mob^- plasmid of about 55 kb. This plasmid encodes resistance to carbenicillin, gentamicin, streptomycin, and sulfanomides (26).

Plants used in this study and their cultivation. Seeds of soybean cv. Toans and bean cv. Blue Lake were surface sterilized in 10% NaOCl for 10 min and rinsed thoroughly with sterilized distilled water (9). Two to three soybean or bean seeds were planted 1 in. (2.54 cm) deep in sterilized potting soil in 4-in. (ca. 10-cm) plastic pots. After germination, seedlings were thinned to two plants per pot. The sterility of the soil was

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, 307 Life Sciences East, Oklahoma State University, Stillwater, OK 74078. Phone: (405) 744-7180. Fax: (405) 744-6790. Electronic mail address: rum67@vms.ucc.okstate.edu.

confirmed just before planting by shaking 2 g of soil in 25 ml of *Pseudomonas* minimal medium (21) and plating serial dilutions on Luria plates (Luria broth containing 1.3% agar). All pots were watered daily with sterilized distilled water. Eight days after planting, 76% of the beans and 85% of the soybeans had germinated.

Inoculation of plants and determination of leaf colonization. Cultures of RM2140 and PAO515 grown in Luria broth (21) were centrifuged at 4,300 × *g* at room temperature for 10 min, washed in 0.85% saline, and resuspended in a volume of saline equal to the original volume of culture medium. Inoculation of leaf surfaces was done by the method of Farrand (7). Briefly, the inoculum of one or the other bacterial strain was dribbled onto the upper surface of every leaf (until the leaves were saturated) of eight plants, using a sterile Pasteur pipet to prevent any bruising or wounding of plant leaves. Care was taken to air dry each leaf before the next leaf was inoculated. The plants were enclosed in sterile (drenched in 10% NaOCl solution and then rinsed in sterile distilled water) clear plastic bags to maintain high humidity and prevent contamination. Recoverable CFU were assayed for 11 days following inoculation by harvesting two inoculated leaves every other day with a flame-sterilized forceps and shaking each leaf in 10 ml of *Pseudomonas* minimal medium buffer for 30 min. The leaves were removed and serial dilutions, prepared in *Pseudomonas* minimal medium, were plated on Luria plates. Since spontaneous or stress-stimulated induction of the lytic response of the F116 prophage resident in PAO515 would lead to the release of infectious viral particles on the leaf surface (14), the rate of accumulation of F116 PFU was determined by plating the serial dilutions on a lawn of the susceptible host strain PAO1 as described previously (21). Rate-of-change constants ($k_{\log_{10}}$) for bacterial and phage populations were calculated from the formula $k_{\log_{10}} = \log_{10}(C_t/C_0)/t$, where C_0 is the number of CFU or PFU at the time of inoculation and C_t is the number of CFU or PFU recovered at t hours.

Transduction on leaf surfaces. Transducing lysates of bacteriophage F116 virions were not introduced directly onto the plant leaves, but the nalidixic acid-resistant F116 lysogen PAO515 was used as the source of virus particles in planta. This strain also served as the recipient of transduced DNA. RM2140 was used as a plasmid donor, and transduction of the Tra$^-$ Mob$^-$ Rms149 plasmid (26, 27) was scored as an indicator of transduction in planta. The antibiotics carbenicillin and nalidixic acid, each at a concentration of 500 μg/ml, were routinely used for selection.

To determine whether transduction could be observed on the lamellar surfaces of leaves, RM2140 and PAO515 were inoculated onto eight plants each of soybeans and beans 19 days after planting as described above. The two cultures of *P. aeruginosa* were mixed in a 1:1 ratio and dribbled onto the leaves of the test plants with a Pasteur pipet. In addition to these experimental plants, the upper surfaces of the leaves of four bean plants and four soybean plants were inoculated with RM2140 alone, and four additional plants of each species were inoculated with PAO515 alone. These plants served as controls and allowed us to monitor growth throughout the experiment. Immediately after inoculation, the inocula for all plants were tested for the presence of transductants by plating on Luria plates supplemented with nalidixic acid and carbenicillin. No colonies were observed, suggesting that transduction did not take place prior to inoculation of the plants. All plants were enclosed in sterile clear plastic bags and incubated on laboratory benches during a 16-h photoperiod. All plants were grown and maintained at daytime temperatures of 27 ± 3°C and nighttime temperatures of 18 ± 3°C. Two methods were used to examine transduction in planta.

(i) Qualitative method. Following inoculation, sampling was done on alternate days for a 10-day period. At each sampling period, two leaves from each crop were excised at random with a flame-sterilized forceps and placed on Luria plates supplemented with nalidixic acid and carbenicillin (to select for transductants), with the inoculated surface in contact with the media. A 2.5-ml portion of lambda top agar which contained 10 g of tryptone, 5 g of NaCl, and 6.5 g of agar per liter of distilled H_2O (21) was poured over each leaf to increase the contact between the leaf and the surface of the agar plate. Each plate was incubated at 37°C for 2 days before removal of the leaf and scoring for the presence or absence of transductant colonies.

(ii) Quantification method. In order to quantify transduction in planta, two leaves were excised at each sampling time. Each leaf was placed in 10 ml of *Pseudomonas* minimal medium buffer containing nalidixic acid and shaken vigorously for 1 h. The nalidixic acid was added to prevent both phage production and replication of plasmid donor organisms (26, 27). This solution was then filtered through a 0.45-μm-pore-size cellulose nitrate filter and the filter was placed on Luria plates supplemented with nalidixic acid and carbenicillin to determine the relative concentrations of the transductants (26, 27). These procedures have been previously shown to reduce the number of transductants formed during the sampling procedure (as opposed to in planta) to below the level of detection (26, 27). The number of CFU of transductant and parental phenotypes and the number of PFU of bacteriophage F116 were scored as outlined by Saye et al. (26, 27).

All presumptive transductant colonies were further confirmed by molecular analysis of the plasmid DNA present in the cells. Plasmid DNA from the presumed transductants was isolated by the alkaline lysis method (19) and cleaved with *Eco*RI. Restriction endonuclease patterns were assessed on a 0.7% agarose gel (26) and compared with that of Rms149 DNA extracted from RM2140.

Bidirectional gene transfer on leaf surfaces. Three plants each of beans and soybeans were grown per pot to simulate high plant density. They were grown and inoculated as described above except that RM2140 (the plasmid donor) was inoculated onto the soybean plants and PAO515 (the lysogenic recipient of transduced DNA) was introduced onto the bean plants. Control plants were inoculated as described above except that bean and soybean plants present in the same pot were enclosed in separate plastic bags. Sampling and plating protocols were identical to those described above. The control plants were used to monitor the growth of the parental strains.

Qualitative and quantitative examinations of the transmission of genetic material among bacteria resident on adjacent plants were carried out as described above. Incubation was carried out for 11 days, with samples taken every other day.

RESULTS AND DISCUSSION

Colonization of leaf surfaces. *P. aeruginosa* was inoculated onto bean and soybean plants as described in Materials and Methods. Recoverable bacterial CFU and phage PFU were monitored over an 11-day period. A remarkably steady exponential decline occurred over the time course of the experiment (Fig. 1). However, viable bacteria were observed even on the 11th day of incubation. The presence of microbes at the end of the test period indicates that *P. aeruginosa* survived to that time on the surfaces of plant leaves. Many environmental and biological factors (such as leaf age) affect bacterial popu-

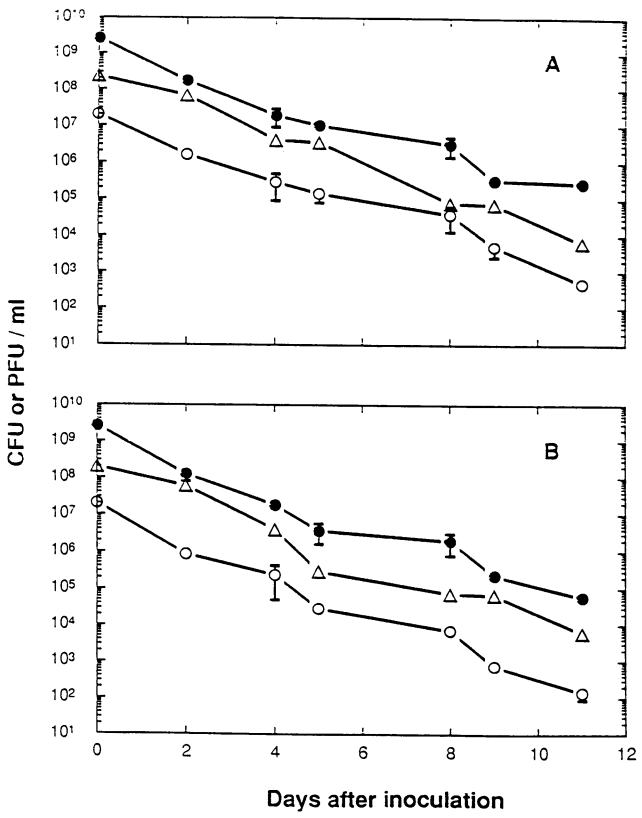

FIG. 1. Populations of bacteria and phages on bean leaves (A) and soybean leaves (B) sampled 0 to 11 days after inoculation. Symbols: ○, donor (RM2140; $k_{\log_{10}} = -0.022$ on beans and -0.018 on soybeans) CFU; ●, recipient (PAO515; $k_{\log_{10}} = -0.021$ on beans and -0.019 on soybeans) CFU; △, bacteriophage F116 ($k_{\log_{10}} = -0.017$ on beans and -0.016 on soybeans) PFU. Error bars represent the standard errors of the means from two leaves.

lations over time (13, 18). Bacterial numbers have been shown to range from nondetectable levels to nearly 1.5×10^5 CFU per leaf on individual cherry leaves (2) and to vary by as much as 1,000-fold on the leaves of corn (10). This variation may lead to spotty colonization and spatial variation in population densities.

Transduction on leaf surfaces. Saye et al. (26, 27) demonstrated that environmental lysogens of generalized transducing bacteriophages can serve as effective reservoirs of transducing phages and as effective recipient bacteria for transduced DNA in situ in aquatic ecosystems. Morrison et al. (24) observed the transduction of *P. aeruginosa* in a freshwater environment in a study using phage F116.

We wished to test whether plant surfaces would support genetic transduction. Plants were inoculated with genetic donors and lysogenic recipients as described above. Our first experiments were qualitative in nature in that we determined only the presence or absence of transductants on leaf surfaces by blotting the leaf surfaces onto selective agar medium. Following inoculation, transductants were observed on 90% (18 of 20) of the leaves studied, suggesting a very high level of occurrence of transduction. All presumptive transductant colonies were picked and regrown on Luria plates supplemented with nalidixic acid and carbenicillin to confirm their phenotypes. The frequency of spontaneous mutation of PAO515 to carbenicillin resistance (assayed from control plants) was below the level of detection. The numbers of transductant colonies found on leaf surfaces generally increased with the time of incubation on the plant. However, the numbers of transductants varied, demonstrating the random occurrence of transduction on plant leaf surfaces. These data suggested that the number of transductants observed was dependent on the conditions of incubation in planta and was not an artifact of the sampling procedure.

TABLE 1. Effect of mixed genetic population on transduction

Plant[a]	Day	No. of transductants (CFU)/ml		No. of transductants/10^7 recipients	
		Leaf no. 1	Leaf no. 2	Leaf no. 1	Leaf no. 2
Bean	0	NO[b]	NO	<0.1	<0.1
	2	7	13	0.5	0.9
	4	14	14	16	15
	5	14	17	18	21
	8	>50	>50	>350	>360
	9	3	3	94	83
	11	3	2	100	67
Soybean	0	NO	NO	<0.1	<0.1
	2	3	2	0.4	0.3
	4	NO	NO	<0.1	<0.1
	5	34	47	213	294
	8	22	26	275	325
	9	3	3	130	134
	11	4	5	833	1,042

[a] Two randomly selected leaves from each crop were used at each sampling time as described in the text.
[b] NO, not observed.

Quantification of transduction. In an attempt to quantify transduction in our model phylloplane system, we modified our experimental procedure as described above. The frequencies of transductants, donor and recipient bacteria, and bacteriophage F116 virions were determined by sampling leaves every other day over an 11-day period. No transductants were observed on control plants (i.e., those inoculated with only one of the parental strains). With plants inoculated with both parental strains, transductants were observed by the second day of incubation on the leaf surface, and their frequency increased during the first 8 days of incubation before finally declining on day 9 (Table 1). The apparent decline in the number of transductants was probably due to the fact that *P. aeruginosa* populations of all phenotypes had declined during incubation on the surfaces of these leaves (Fig. 1) and not to any intrinsic negative selection for the transductant phenotype. This assumption is supported by the fact that the frequency of transductants as a function of the recipient population continued to increase during the entire sampling period (Table 1). Similar observations were reported by Saye et al. (26, 27) for in situ transduction studies with samples from a freshwater lake.

Presumptive transductants were confirmed by genetic and molecular analysis. Plasmid DNA from the presumed transductants was isolated and cleaved with the restriction enzyme *Eco*RI. Restriction endonuclease patterns were assessed by agarose gel electrophoresis and compared with that of Rms149 DNA extracted from RM2140. Each of the transductants carried a plasmid with a restriction pattern identical to that of Rms149.

Under laboratory conditions with the use of cell-free lysates, the frequencies of transduction of Rms149 by F116 have been reported to be 0.3×10^{-7}, 0.9×10^{-7}, and 3.3×10^{-7}

transductants per PFU at multiplicities of infection of 2, 0.2, and 0.02, respectively (27). In laboratory studies in which lysogenic recipients were incubated with nonlysogenic donors, transduction of Rms149 was observed to plateau after 48 h at frequencies of 10^{-4} to 10^{-5} transductants per recipient bacterium (26). However, results for an environmental test chamber incubated in a freshwater lake indicated that transduction had plateaued within 48 h of incubation in situ at frequencies of 10^{-5} to 10^{-6} transductants per recipient bacterium (26). These frequencies are very similar to those which we observed. Transduction on plant leaves plateaued after 4 to 5 days at frequencies of 10^{-5} to 10^{-6} transductants per recipient bacterium (Table 1). Thus, it appears that frequencies of transduction are similar in these two environmental habitats, at least for this model system. However, plateau values for transductant-to-recipient ratios were not reached as rapidly in the in planta system. This may indicate that genetic interaction among bacteria distributed on the solid leaf surface is inhibited by spatial separation and reduced migration compared with the interactions possible in the more liquid environment of the freshwater lake.

Gene transfer on leaf surfaces as a result of the bidirectional movement of microbes between adjacent plant surfaces. Planting patterns in our agricultural microcosms were devised to simulate the plant-to-plant (and thus leaf-to-leaf) contact that occurs under field conditions because of dense planting by farmers, high wind velocities, and various mechanical operations performed during crop growth. In these experiments, the donor was inoculated onto the leaves of one type of plant and the recipient was inoculated onto the leaves of the other. We then measured the numbers of transductants on the leaves of both types of plants.

Qualitative experiments in which leaves were placed directly onto transductant-selective medium revealed that transductants could be observed on both bean and soybean leaves (15 of 20) sampled over a 10-day incubation period. These results suggested that the migration of microbes or bacteriophages occurred bidirectionally between adjacent leaf surfaces, resulting in genetic exchange. The relative frequencies of transductants observed were variable at different sampling times, probably because (i) different leaves were sampled at each time and (ii) there was variability in the extent of contact between the leaves.

We then repeated these experiments using the quantitative method described above. The numbers of transductants observed were generally lower in these experiments (Table 2) than in experiments in which both parents were introduced onto the same leaf (Table 1). However, transductants were consistently observed (Table 2), with frequencies commonly ranging from 10^{-6} to 10^{-7} transductants per recipient.

The formation of transductants under the conditions of these experiments requires the movement of either parental bacteria, bacteriophages, or transductants between plants. Unfortunately, the experiments cannot distinguish between these alternatives. In any case, the data obtained demonstrate that movement and reassortment of genetic information between and among bacterial communities resident on adjacent plants can take place and that these populations are not necessarily isolated genetically.

Implications for gene transfer in planta. Our experiments clearly demonstrate the potential for virus-mediated gene transfer among bacteria residing in the phylloplane. They indicate that transduction is a viable mechanism for the spread of genetic elements among microbes on plant lamellar surfaces. Therefore, transduction has the potential to facilitate the evolution of plant-associated microbes and expand the genetic diversity and complexity of the gene pool available to microbial populations of agricultural importance. It may increase the distribution of antibiotic and metal resistance determinants in natural plant-pathogenic populations and must be considered a potential mechanism for the transmission of genetic elements among microbial populations of agricultural significance.

TABLE 2. Transduction through bidirectional movement of bacteria

Plant[a]	Day	No. of transductants (CFU)/ml		No. of transductants/10^7 recipients	
		Leaf no. 1	Leaf no. 2	Leaf no. 1	Leaf no. 2
Bean	0	NO[b]	NO	<0.1	<0.1
	2	3	1	0.1	0.05
	4	2	5	0.7	2
	5	4	6	3	5
	8	2	3	4	6
	9	4	3	125	94
	11	2	2	83	84
Soybean	0	NO	NO	<0.1	<0.1
	2	1	1	0.06	0.07
	4	NO	NO	<0.1	<0.1
	5	3	5	5	9
	8	2	4	6	13
	9	1	2	43	100
	11	1	3	145	462

[a] Two randomly selected leaves from each crop were used at each sampling time as described in the text.
[b] NO, not observed.

Our experiments were conducted in a nearly sterile environment; hence, the transduction frequencies we observed are likely to be higher than would be observed in the presence of the natural microflora of plant leaf surfaces. We can, however, speculate that transduction will take place when the environmental microbiota is present on the leaf. Saye et al. (26) reported transductants in the presence and absence of natural communities of microbes in freshwater environments. In their experiments, the numbers of transductants recovered were highest when experiments were conducted in the absence of the natural microbial community. However, when the number of transductants was normalized to the frequency of recipient bacteria present (20), the ratio of transductants to recipients was constant whether the natural bacteria were present or absent (26).

Transduction may be a particularly important mechanism in situ since the transduced genes may reside on the donor bacterium's chromosome as easily as on a conjugatively proficient plasmid (14). Transduction of chromosomally encoded alleles has been observed at frequencies similar to transduction frequencies of plasmid DNA in aquatic environments (24, 27). Thus, transduction may have a highly significant impact on the evolution of bacterial species and the transfer and reassortment of chromosomal genetic elements. It clearly must be considered an important variable in the genetic ecology of natural populations of bacteria.

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